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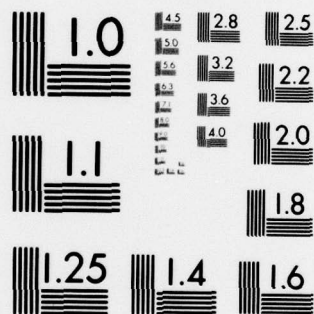
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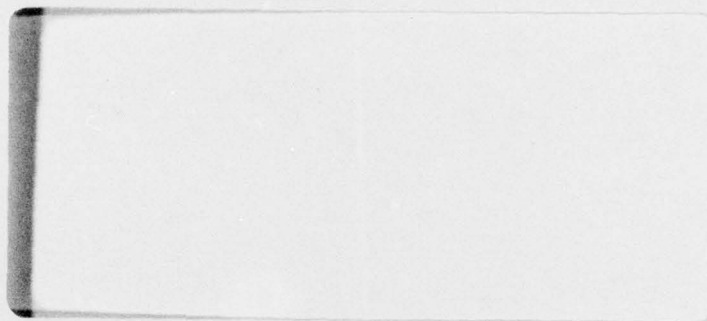


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## ABSTRACT

Work was continued on the development of a rapid method for the counting of coliform bacteria, based on the hydrolysis of a fluorogenic substrate by the enzyme  $\beta$ -D-galactosidase. The procedure was extended to differentiate between total coliforms and fecal coliforms by elevating the test temperature from 35°C to 44.5°C. The effect of various concentrations of chlorine on the coliform contained enzyme  $\beta$ -D-galactosidase was also investigated to confirm that the fluorescence technique was comparable to the standard multiple tube fermentation technique.

Work was also continued on evaluating two bacterial concentration techniques; continuous flow centrifugation and stirred cell filtration. Neither technique was fully investigated.

Preliminary experimentation was conducted on a procedure to eliminate bacterial concentration, yet permit detection and quantitation down to the 2 bacteria per ml level. This procedure involved the microencapsulation of bacteria in liquid membranes prior to passage through a fluorescence flow cytometer.

Validation of the rapid coliform-counting procedure for the examination of field-collected water samples was incomplete, due to winter weather conditions and the late arrival of continuous flow centrifugation components. Semiautomation of the fluorescence microscope, by the addition of a scanning stage, photomultiplier and photometer, doubled the fluorescent droplet counting, and detection rate.

## FOREWARD

This report has been prepared for the Office of Naval Research (ONR), the David W. Taylor Naval Ship Research & Development Center (DTNSRDC) and the U.S. Army Mobility Equipment Research & Development Command (MERADCOM) in accordance with the requirements of Contract N 00014-78-C-0713. The period of performance of the contract was to be September 1, 1978 to August 30, 1979. However, the project was placed in a holding mold due to Naval Materiel Command cuts in Office of Naval Research FY 1979 funding. This report describes the progress to March 30, 1979.

The objective of the program is to extend BioResearch's rapid coliform detection procedure from total to fecal coliforms, demonstrate the feasibility of the procedure for counting coliforms in a variety of field samples, confirm the feasibility of counting coliforms at the 2 per ml level and to initiate the development of an instrumentation system for the automated detection of coliform bacteria.

The program was conducted by the Bioelectrochemistry Division of BioResearch, Inc. The technical program manager was Dr. A.M. Cundell assisted by Janet E. Porter and Anna M. Pisani. Eugene Findl, Director of the Bioelectrochemistry Division, directed the overall effort.

Technical monitors for the program were Dr. A.J. Emery, Jr. (ONR), Dr. S. Finger (DTNSRDC) and Gerald Eskelund, (MERADCOM). Their assistance and guidance is gratefully acknowledged.

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## GLOSSARY

Total Coliforms - aerobic and facultative anaerobic gram-negative, non-spore forming, rod-shaped bacteria that ferment lactose with gas production within 48 hours at 35°C. Include members of genera Klebsiella, Enterobacter, Escherichia and Citrobacter.

Fecal Coliforms - coliforms that ferment lactose, with gas production, within 24 hours at 44.5°C. More selective for bacterial inhabitants of the human gastrointestinal tract, e.g., Escherichia coli than total coliforms.

Enterobacter aerogenes - non fecal coliform with the ability to ferment lactose at 35° but not 44.5°C.

Aeromonas hydrophilia - an oxidase positive, gram-negative rod shaped bacteria with a variable ability to ferment lactose at 35°C.

Fluorochromasia - ability of a mammalian cell or bacterium to intracellularly accumulate fluorescein from the hydrolysis of a fluorogenic substrate.

Flow Cytometer - an instrument that measures the number of cells, cell size, (light scattering) and fluorescence of individual cells in liquid suspension as they pass, in single file, through an optical path or sensing zone. Information on the cell number, cell type, constituent level, amount of dye and cell size is obtained at a rate of tens of thousands of cells per minute.

Free available chlorine - chlorine existing in water as hypochlorous acid. Characterized by rapid color formation with orthotolidine or diethyl p-phenylene diamine.

Chlorine demand - the difference between the applied chlorine and the residual available chlorine in an aqueous medium under specific conditions and contact time. Since chlorine is an oxidizing agent it will be consumed by organic material and ammonia within the medium.

## ABSTRACT

Work was continued on the development of a rapid (on the order of one hour) method for the counting of coliform bacteria, based on the hydrolysis of a fluorogenic substrate by the enzyme  $\beta$ -D-galactosidase. The procedure was extended to differentiate between total coliforms and fecal coliforms by elevating the test temperature from 35°C to 44.5°C. The effect of various concentrations of chlorine on the coliform contained enzyme  $\beta$ -D-galactosidase was also investigated to confirm that the fluorescence technique was comparable to the standard multiple tube fermentation technique.

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Validation of the rapid coliform-counting procedure for the examination of field-collected water samples was incomplete, due to winter weather conditions and the late arrival of continuous flow centrifugation components. Semiautomation of the fluorescence microscope, by the addition of a scanning stage, photomultiplier and photometer, doubled the fluorescent droplet counting, and detection rate.

## INTRODUCTION

There is a need for a real time coliform-counting procedure to monitor coliform bacteria in discharge wastewater, surface water, industrial process water and potable water. Present standard methods of counting coliforms are (a) the multiple-tube fermentation technique (b) the membrane filter technique. These procedures require incubation of inoculated media for at least 24 hours, to allow for colony formation on membrane filters or fermentation of lactose in broth tubes (American Public Health Association, 1975). Disadvantages of the standard methods are the necessities of maintaining sterile technique, the consumption of large quantities of media and laboratory ware, the amount of labor expended in the procedures, and the time delay in obtaining coliform numbers. (In many instances, decisions on water quality should be made without delay to protect public health and/or prevent environmental impact.)

Criteria for an ideal rapid coliform-counting procedure include:

- a) specificity for fecal coliforms,
- b) test completion is less than an hour,
- c) results comparable to those obtained with multiple-tube fermentation and/or membrane filtration techniques,
- d) good reproducibility and accuracy,
- e) a lower limit of a single coliform,
- f) amenability to automation,
- g) equipment priced in the range of the average testing laboratory,
- h) procedure should be technically simple, and
- i) a potential for field use.

Obviously, it is a tall order to develop a coliform-counting procedure that will satisfy all these criteria. Our approach to meeting these criteria is the detection of an enzyme system, specific to coliform bacteria, as the basis of a counting procedure, that permits organism counting without the need for growth. The feasibility of a rapid coliform detection procedure, based on the hydrolysis of a fluorogenic substrate by the inducible enzyme  $\beta$ -D-galactosidase, was demonstrated under Contract N00600-77-C-1163. This procedure represented an adaptation of the method used for the detection of single molecules of  $\beta$ -D-galactosidase in Escherichia coli (Rotman, 1961). The procedure involved the induction of  $\beta$ -D-galactosidase, within coliforms, using the powerful inducer isopropyl thio  $\beta$ -D-galactopyranoside (IPTG). Cell suspensions ( $10^5$  to  $10^8$  organisms per ml) of the induced coliform bacteria were dispersed onto modified microscope slides, containing a layer of silicone oil, using an aerosol sprayer. Within the resultant droplets (5 to 50  $\mu$ m diameter), a fluorogenic

substrate, fluorescein di  $\beta$ -D-galactopyranoside (FDG)( $5 \times 10^{-4}M$ ) is hydrolyzed by coliform bacteria, when the slide is incubated at  $35^{\circ}C$ . Liberated fluorescein, is concentrated within individual oil encapsulated droplets, enabling them to be viewed and counted using a fluorescence microscope (excitation filter BG 38, barrier filter yellow 510). A standard curve of the percentage of fluorescent droplets per field of view versus the coliform cell density was established and used to count coliform bacteria in cell suspension of unknown cell density.

A limitation to the rapid coliform-counting procedure demonstrated in the feasibility study was its applicability to coliform cell densities below  $10^5$  organisms per ml. Three approaches were to be investigated under Contract N 00014-78-C-0713, to count coliform cell densities below  $10^5$  organisms per ml, a) concentration by continuous flow centrifugation, b) concentration by stirred cell membrane filtration and c) the use of a fluorescence flow cytometer.

## TECHNICAL DISCUSSION

Details of the revised instrumentation developed, fluorescence analysis procedure modifications and new techniques investigated during the contractual period are discussed herein.

### Instrumentation

A Zeiss Standard 15 microscope, equipped for transmittance and incident fluorescent light observation, was used in this investigation. Illumination was provided by a 12 volt halagen quartz light source, with a BG 38 excitation filter and a 510 barrier filter. The light source, in the transmittance mode, was focused on a modified microscope slide, using an oil immersion darkfield condenser. Optics were a 10X Achromat objective lens and paired KPL 12.5X eyepieces. The 510 barrier filter was included in the left eyepieces so that the fluorescence was superimposed in the dark field image to aid the counting.

Our microscope was further modified to include a semi-automatic mode of counting fluorescent droplets. A Zeiss Scanning stage (10  $\mu$ m steps) with a motorized joy stick control was attached to the microscope in place of the existing stage. The scanning stage permitted serial examination of fields of view in a systematic and rapid fashion.

A Farrand microscope spectrum analyzer, consisting of a grating monochromator, photomultiplier tube and photometer, was attached to the phototube of the microscope. Photoemission from individual fluorescent droplets is collected and measured as an intensity at the selected wavelength (525nm). This reading can be taken directly from the photometer, amplified and fed to a strip-chart recorder, or processed through a digital readout system.

A JCF-Z continuous flow centrifuge core was purchased to be used in our existing Beckman model J-21 C centrifuge. To drive coliform cell suspensions through the core, a rotor pump (Econ-o-pump P-1, Eastern Industries) was employed. Assembly of the continuous flow centrifuge is outlined in Figure 1. Since *Escherichia coli* has a sedimentation coefficient ( $5 \times 10^{13}$ ) of 3600, the recommended flowrate at 15,000 rpm (12,000g) was 200 ml per minute. At this flowrate the residence time (5 min/liter) is sufficient for a sufficient number of bacterial cells to be spun down and collected as pellets in the collection canoes.

## FILTRATION & CONCENTRATION PROCEDURE - CONTINUOUS FLOW CENTRIFUGATION

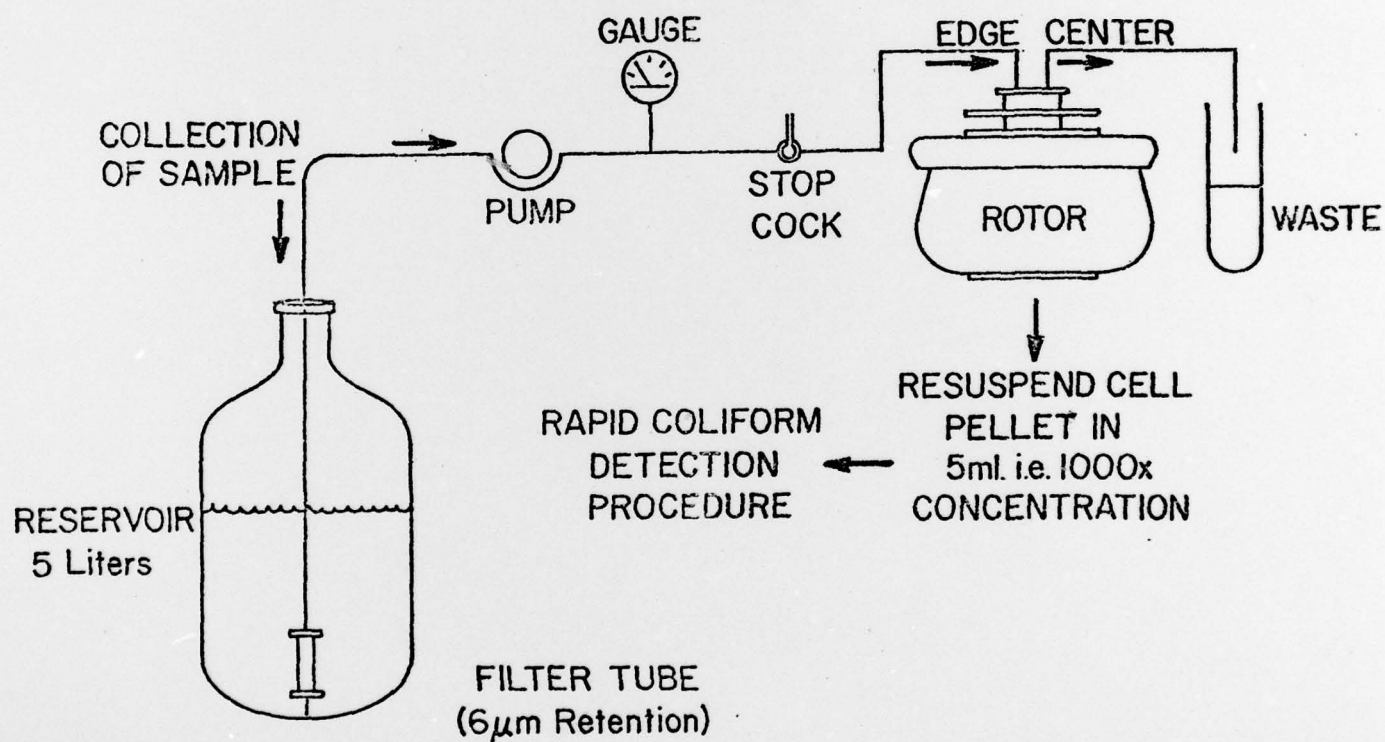


Figure 1

## Extension of the Rapid Coliform Detection Method to Fecal Coliforms

Since the presence of fecal coliforms in a water sample is more indicative of fecal contamination than total coliforms, it was an obvious step to extend the rapid coliform detection procedure from total to fecal coliforms. Two strategies were followed, namely, the addition of specific salts to the coliform detection medium and the use of an elevated incubation temperature. Test organisms employed in this stage of the project were Escherichia coli Neotype ATCC #11735 and Enterobacter aerogenes ATCC#13048. The rapid coliform detection procedure was modified by 1) the inclusion of 0.15% bile salts in the induction medium to determine its effect on  $\beta$ -D-galactosidase activity and 2) the use of 44.5°C instead of 35°C as an incubation temperature.

Inclusion of bile salts within the induction medium did not interfere with the  $\beta$ -D-galactosidase induction or activity. Bile salts are known inhibitors of gram positive bacteria but they did not assist in the differentiation of total and fecal coliform bacteria using the fluorescence technique. In contrast, elevated temperature leads to differentiation using the fluorescence coliform detection procedure. No fluorescent droplets were observed with slides sprayed with an induced Enterobacter aerogenes cell suspension and fluorogenic substrate when incubated for 15 minutes at 44.5°C. Using a lower incubation temperature, i.e., 35°C, fluorescent droplets were readily apparent.

Further experimentation was conducted to confirm the suitability of 44.5°C as an incubation temperature for the detection of E. coli by virtue of its  $\beta$ -D-galactosidase activity. A recent publication of Dockins and McFeters (1978) described their investigation of the effect of temperature upon  $\beta$ -D-galactosidase activity in cell-free extracts of fecal and nonfecal coliforms. They found a precipitous decline in enzyme activity above 35°C for a field-isolated fecal coliform. In contrast, we found that the specific activity of  $\beta$ -D-galactosidase (substrate o nitrophenol galactopyranoside ONPG) increased from 0.124 to 0.124 and to 0.162 at 30°, 35° and 45°C respectively for E. coli Neotype ATCC #11775. Elevated incubation temperature also increased the intensity of the fluorescent droplets during the 15 minute incubation period.

A series of experimental runs (5 runs at each of 5 coliform cell densities) were completed to demonstrate the linearity of the relationship between the percentage of fluorescent droplets per field of view and the fecal coliform cell density as measured by plate counts on nutrient agar (Figure 2). The validity of the plate counts was tested by constructing a standard curve of the E. coli cell density measured by the multiple-tube fermentation technique (Figure 3). These experiments clearly established the applicability of the rapid coliform detection procedure to the quantification of fecal coliforms.

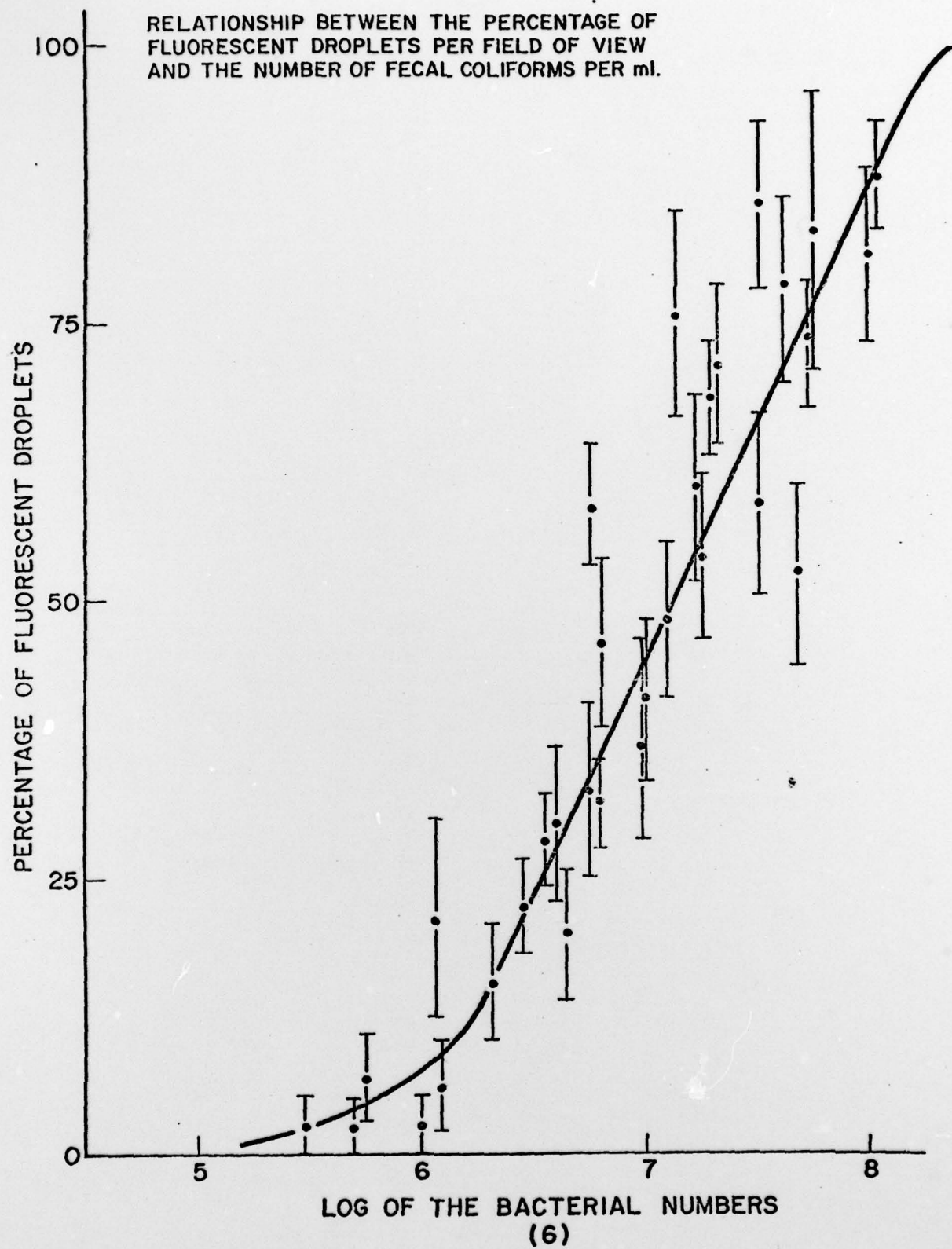


FIGURE 2

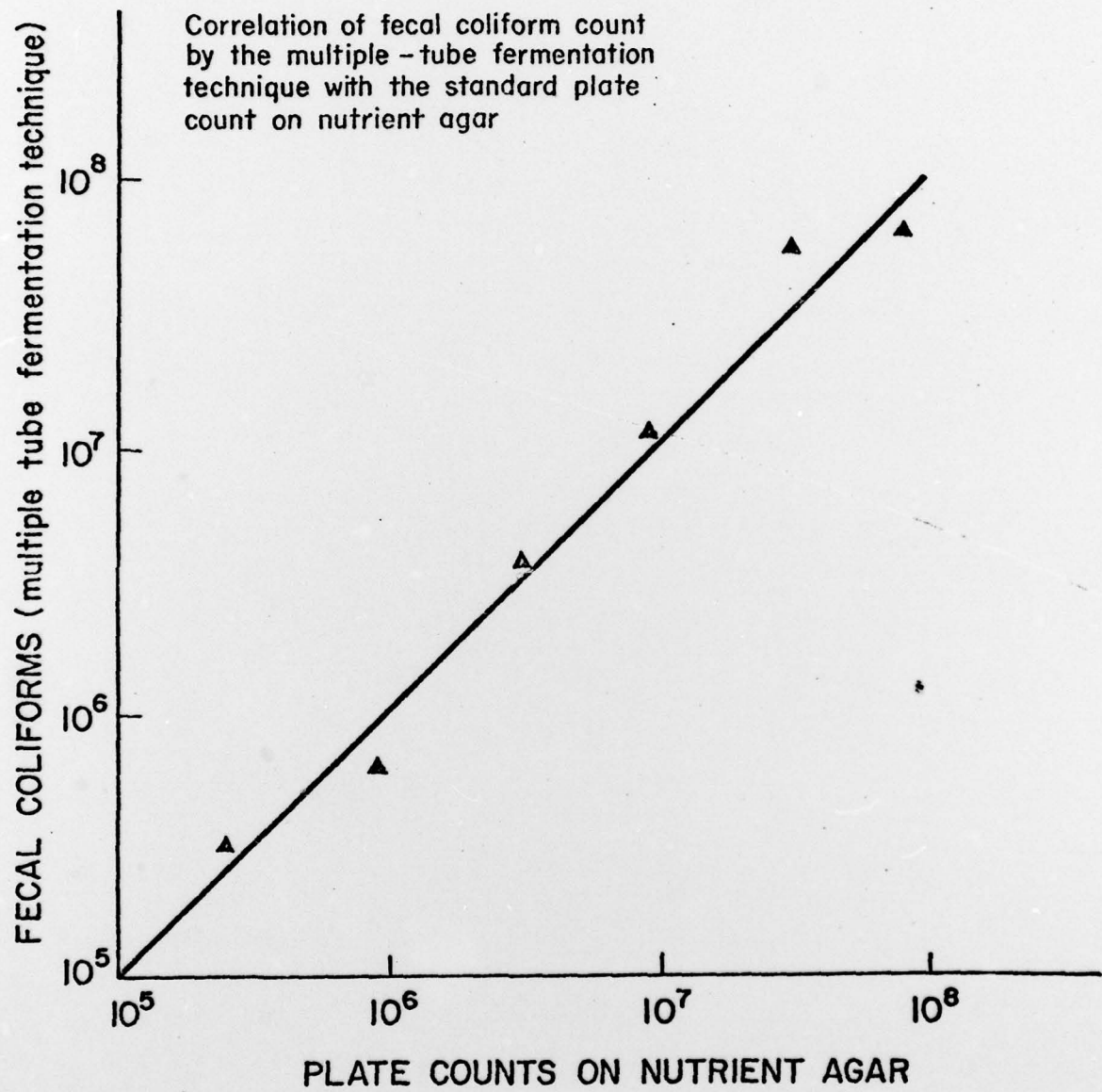


FIGURE 3

### Effect of Chlorination on the Rapid Coliform Detection Procedure

Chlorine is an effective bacterial disinfectant. Exposure of *Escherichia coli* to 0.05 ppm of available chlorine for 1 second at 25°C will result in a 100% loss in viability (Dychdala, 1977). However, the effectiveness of chlorine as a biocide depends on a number of factors including pH, temperature, chlorine concentration, presence of ammonia, and presence of organics in the solution.

In water distribution systems, a residual chlorine concentration of about 0.5 ppm of chlorine is maintained to kill bacterial pathogens that may enter the system and contaminate the water. Coliform bacteria, exposed to low levels of chlorine, are capable of so-called repair when transferred to a rich nutritive medium. Since the rapid coliform detection procedure is based on the  $\beta$ -D-galactosidase activity within individual coliform bacteria and not the ability of the bacteria to divide, it is possible that chlorine injured coliforms may be countable in our test procedure but not in the standard methods. The effects of chlorine on the  $\beta$ -D-galactosidase activity within cell suspensions of *Escherichia coli*, was therefore investigated.

To reproduce similar conditions to that found in sewage treatment plants when the final effluent is chlorinated, held and discharged, a cell suspension of *E. coli*, induced for  $\beta$ -D-galactosidase activity, was exposed to 5 ppm of chlorine for 30 minutes. The change in viable cell numbers and enzyme activity was then determined.

*E. coli* were grown overnight in a lactate medium containing  $5 \times 10^{-4}$ M IPTG. Cells were harvested from the medium by centrifugation, washed in phosphate buffer and resuspended in buffer (optical density ~0.1). *E. coli* cells were exposed to sodium hypochlorite (available chlorine about 5 ppm) for 30 minutes at room temperature. The chlorine was then neutralized by the addition of a sodium thiosulphate solution. Two controls were run in parallel; cell suspensions without chlorine and chlorine without bacterial cells. Viable coliform numbers were determined using the multiple-tube fermentation technique. Enzyme activity was assayed by the ONPG hydrolysis method. Free chlorine was measured using the DPD-chlorine colorimetric assay (APHA, 1975). Test results are tabulated below.

TABLE I  
EFFECT OF CHLORINE ON AN E. COLI CELL SUSPENSION

|                  | Free chlorine<br>(ppm) |         | Viable<br>Cell Number |                   | $\beta$ -D-galacto-<br>sidase<br>Activity |         |
|------------------|------------------------|---------|-----------------------|-------------------|---|---------|
|                  | 0 min.                 | 30 min. | 0 min.                | 30 min.           | 0 min.                                    | 30 min. |
| Cells            | ----                   | ----    | $3.5 \times 10^8$     | $5.0 \times 10^8$ | 0.9                                       | 0.8     |
| Cells & Chlorine | 1.40                   | 0.15    | $4.5 \times 10^8$     | 0                 | 0.5                                       | 0.0     |
| Chlorine only    | 3.68                   | 3.64    | ---                   | ---               | ---                                       | ---     |

Results of this experiment demonstrate that the enzyme  $\beta$ -D-galactosidase is sensitive to chlorine. Enzyme activity was rapidly reduced in the zero time cells and chlorine suspension, whereas viable cell numbers showed no such decline. This finding suggests that the enzyme activity may be more sensitive to chlorine than total cell viability. However, it should be noted that the cells had opportunity to repair themselves in the lactose broth used to make the viable counts.

#### Modifications to the Rapid Coliform Detection Procedure

The original rapid coliform detection procedure was not suitable for counting coliform densities below  $10^5$  organisms per ml. Therefore, a number of modifications to the procedure were investigated. These modifications included;

- a) Measurement of fluorogenic substrate hydrolysis by coliform bacteria in solution,
- b) Counting fluorescent bacteria on the surface of a membrane filter with the fluorescence microscope with an incident light attachment and
- c) Counting fluorescent bacteria with a flow cytometer.

Warren and his coworkers (Warren et.al., 1978) developed a technique to count fecal coliform bacteria using ONPG hydrolysis in a colorimetric assay for  $\beta$ -D-galactosidase. [Long term incubation and/or large numbers of coliforms are required.] A similar approach would be to use a fluorogenic substrate like FDG and measure the concentration of fluorescein produced within incubated coliform suspensions. To test the feasibility of measuring the fluorescein produced by the hydrolysis of the fluorogenic substrate FDG within a coliform cell suspension, a standard curve of the absorbance at 493.5 nm against sodium fluorescein concentration was constructed. There was a linear relationship between absorbance and

concentration in the concentration range of  $10^{-4}$  to  $10^{-6}$ M. This finding was encouraging enough to attempt to measure the fluorescein produced by an E. coli suspension on addition of  $2 \times 10^{-4}$ M FDG. Isoamyl alcohol is added to aid the diffusion of the fluorogenic substrate into the bacterial cells and to speed up the FDG hydrolysis. Unfortunately, the presence of the alcohol within the cell suspension caused turbidity within the curvette, so that a spectrophotometric assay was not possible. Alcohol could be omitted from the procedure but this would increase the incubation time.

Another approach investigated was the addition of the fluorogenic substrate to induced E. coli suspensions, incubation of the cells to build up the intracellular fluorescein concentration, followed by filtration to concentrate the bacterial cells on an autofluorescence-quenched Nucleopore membrane filter. The membrane filter was placed on a microscope slide and layered with silicone oil. The surface was inspected using the fluorescence microscope, operated in the incident light mode. Insufficient fluorescein was associated with the individual coliform bacteria to make them clearly discernable from the membrane background. Whether this was a function of the low intensity of the excitation wavelength light or the concentration of the fluorescein was not resolved.

Three concentration procedures for coliform bacteria were evaluated. They were 1) flocculation within aluminum hydroxide or alginic acid flocs, concentrated by centrifugation and resuspension of the pellet in a minimal amount of buffer 2) a filter tube concentration procedure and 3) a membrane filter concentration procedure.

The first procedure was a modification of a virus concentration technique reported by Melnick and his coworkers (Farrah et.al., 1976). Coliform bacteria were added to a 0.003M  $AlCl_3$  solution (pH 3.5) and 1M sodium carbonate added dropwise until the solution reached a pH of 7.5. A finely divided floc of  $Al(OH)_3$  was precipitated adsorbing the bacteria. The floc was collected as a pellet by centrifugation and resuspended in minimal amounts of glycine-sodium hydroxide buffer (pH 10.4). The efficiency of adsorption of the bacteria to the floc was good. However, desorption from the floc by dissolution with glycine-sodium hydroxide buffer led to a serious loss in cell viability. The extremes of pH apparently do not effect virus' but are lethal to bacteria.

In a literature review, we found that Levin and his coworkers (Levin et.al., 1974) used a filter tube concentration technique to concentrate coliform bacteria and then incubated the tubes in broth cultures to enumerate low coliform cell densities. A problem encountered in the procedure was the quantitative removal of the coliforms from the filter tube. Shaking with glass beads and low energy sonication were tried. Poor recoveries (less than 50%) were obtained.

Apparently, the bacteria are retained within the borosilicate glass microfibers of the filter tube and are not readily recoverable. Shaking for periods of ten minutes was superior to 90 seconds sonication. As a removal technique sonication has the danger of disruption of the cells and the filter tube. This technique was therefore eliminated from further consideration.

Experimental work was undertaken with stirred filtration cell concentrator (Amicon Model 52). Problems encountered with this approach were (a) the presence of particulates in coliform suspensions blocked the Nucleopore filter (0.2  $\mu$ m diameter), (b) the poor recovery rate of coliforms due to stickage to the filter and (c) the inability to obtain a continuous flow system with a pressurized stirred cell. On a typical run (500 ml concentrated to 5 ml) considerable numbers of bacteria were attached to the filter and not in the residue. This finding suggested that the cell suspension could be taken down to dryness and the bacteria recovered from the filter by shaking with glass beads in a minimal volume of buffer containing a detergent like Tween 80 to aid the removal of the bacteria. This modification proved to be feasible but the need to recover the bacteria in a minimal volume of buffer decreased the concentration and was time-consuming.

We also investigated the possibility of combining the concentration, induction and fluorogenic substrate hydrolysis sequence within the stirred cell concentrator. A preliminary test was run, where a 500 ml coliform cell suspension was concentrated to 5 ml, acridine orange was added (0.04% concentration) and the suspension incubated for 15 minutes. Bacteria on the membrane filter were counted using epifluorescence microscopy. The original cell suspension contained  $6.5 \times 10^7$  cells (recovery 75%). The difference may be accounted for by the variation in plate counts and direct counts and the adhesion of cells to the sides of the concentration cell. Contact between the acridine orange stained bacteria and the filter surface was good, especially as the filter darkened on exposure to the excitation light.

A similar experiment was run with cesspool waste that had been prefiltered through a filter tube (25  $\mu$ m size retention). Unfortunately, the sample readily clogged the following membrane filter and made bacterial filtration extremely slow at best.

Prefiltration, using filter tubes, is also required for several other approaches. Before a water sample can be concentrated and run through the rapid coliform detection procedure it is necessary to remove particulates that would block the tapered capillary tube used in the aerosol sprayer (diameter 20  $\mu$ m). Some particulates, e.g., diatoms

have the potential for autofluorescence hence, also must be removed.

The passage of a large volume sample through a filter tube (Balston Filter Products, Lexington, Mass.) has the potential of reducing particulates without reducing coliform numbers. Balston filter tubes were tried with 0.2, 2, 8 and 25  $\mu\text{m}$  size retention properties. The percentage of coliforms retained were 99.9, 50.0, 0.0, and 0.0% respectively. This finding suggests that an 8  $\mu\text{m}$  filter tube can be used routinely to remove particulates in a batch or continuous filtration mode, without reducing the coliform count.

#### Automation of the Fluorescent Droplet Counting Procedure

Selection of fields of view and counting of the percentage of fluorescent droplets within the individual fields of view on microscope slides tends to be a subjective and time-consuming activity. The greater the number of fields counted the greater the degree of accuracy and the lower the cell density that can be counted, using the rapid coliform detection procedure. At present, it takes about 10 minutes to manually count the fluorescent droplets in 10 fields of view with a lower detection limit of about  $5 \times 10^5$  organisms per ml. A technique proposed to more rapidly count a greater number of fields was to place a semi-automated scanning microscope stage on our existing microscope, and mount a microscope spectrum analyzer onto the existing phototube (See Instrumentation).

Our approach was to select the most intense fluorescent droplets in a field of illumination, focus on the droplet using a sighting spot of 15  $\mu\text{m}$  diameter, reduce the excitation light intensity at 495 nm to limit fading of the fluorescein and select a suitable sensitivity range to obtain a 70-80% full scale photometer deflection. The sighting spot was then focused on the dark background to verify that the photometer would not respond to the background. The scanning stage was moved along a predetermined path and the number of responses (emission at 525 nm wavelength) above a threshold fluorescence intensity was recorded.

Using this technique it is relatively easy to scan a microscope slide for fluorescent droplets. Methods of collecting data, such as visual reading, digital printout, strip-chart recording, etc. were reviewed. Feeding of the photometer signal to a strip-chart recorder was tried. A series of spikes, height proportional to fluorescein concentration, were obtained during slide scanning (Fig.4). Photometer response time and rate of progress of the scanning stage were limiting factors. Counting rates of fluorescent droplets on the order of 50 per minute were achievable.

# STRIP-CHART RECORDING OF PHOTOMETER RESPONSES

Key: *a.*/ Threshold (non-fluorescent droplets)

*b.*/ One fluorescent droplet

*c.*/ One or more fluorescent droplet

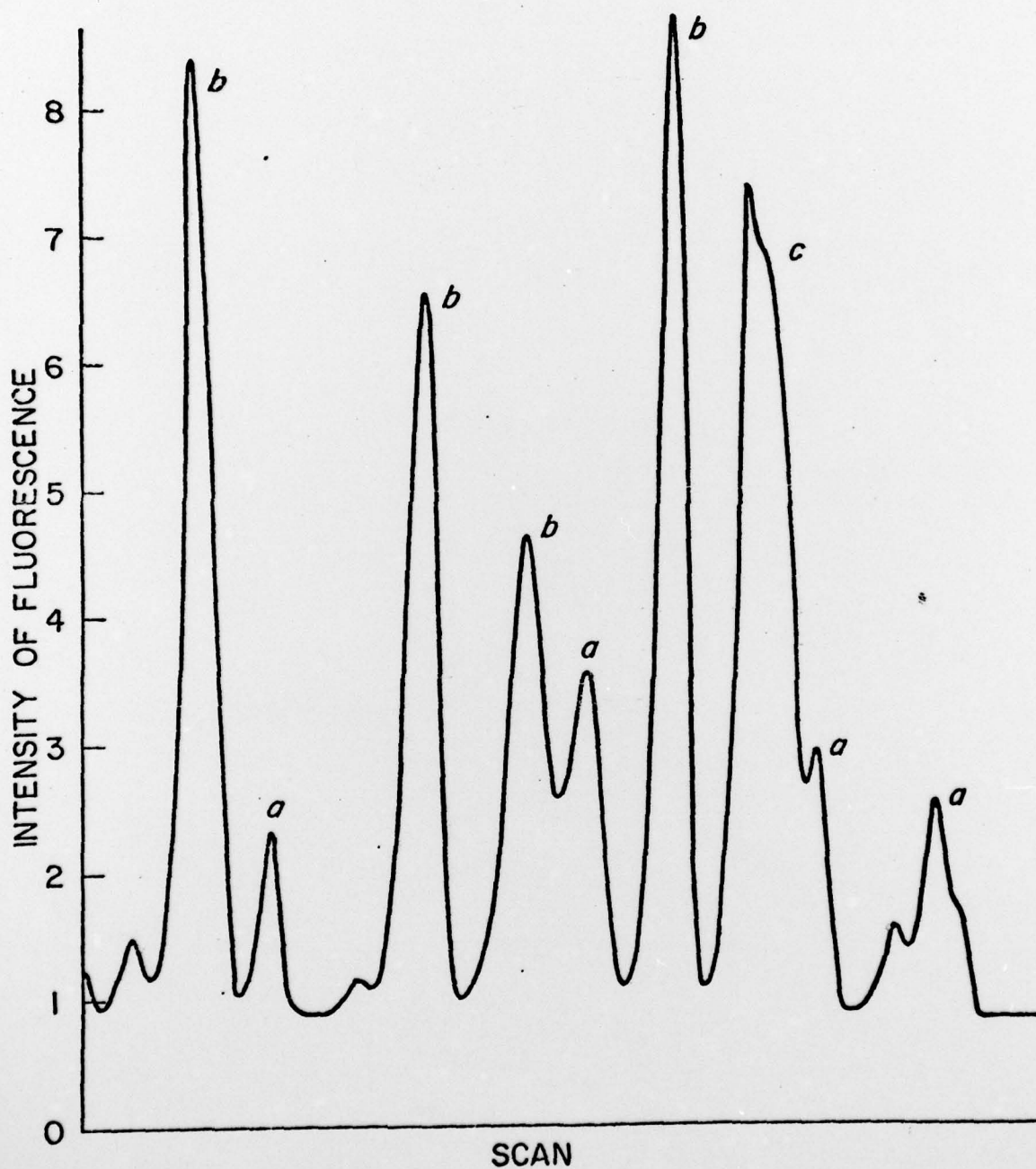


Figure 4

(13)

Hence, it was possible to count 1000 droplets in 20 minutes. This rate was only twice that obtainable using the manual system but the semi automated system can be used continuously over a working day without operator eye fatigue; a distinct advantage when handling a large number of samples.

A problem encountered with this approach was how to concurrently count the total numbers of droplets to determine the percentage of fluorescent droplets. A possible solution suggested itself, namely, spiking the bacterial suspension with a minimal amount of free fluorescein to ensure that all droplets give a response with the instrument. This approach was tested and found to be feasible.

To determine whether the micorphotometer system could be used to measure the concentration of fluorescein in the fluorescent droplets, solutions of fluorescein sodium were sprayed onto separate slides and stored in the dark until they were examined. Intensity readings were made on 10 droplets of approximately the same size (15-20 $\mu$ m) for each concentration ( $10^{-6}$ ,  $5 \times 10^{-6}$ ,  $10^{-5}$  and  $5 \times 10^{-5}$ M fluorescein Na). A linear relationship between the fluorescence intensity and the fluorochrome concentration was observed. Several minor problems developed, namely, fading of the fluorescein, size selection, and the variation in a plane of focus of the droplets.

The influence of droplet size on the fluorescent intensity was investigated. Intensity was proportional to the sighting spot diameter for a constant droplet diameter. Examination of a range of droplet sizes (15-20  $\mu$ m and 25-30  $\mu$ m and 25-30  $\mu$ m diameter) with slits of 0.9 mm diameter (10  $\mu$ m field size) was found to give the same average fluorescence intensity. The rate of fading of fluorescent droplets, with respect to time, was dependent on the light intensity. Selection of the lowest usable excitation light intensity kept fading to a minimum.

A second and more promising approach to automation of the fluorescence rapid coliform detection approach eliminates the necessity of pre-concentrating bacterial samples. This approach involves the use of a fluorescence flow cytometer (rather than a fluorescence microscope) and the microencapsulation of a 1 ml bacterial sample using liquid membranes.

Liquid membranes are water-immiscible emulsions consisting of an oil phase composed of a surfactant, e.g., Span 80, a membrane strengthening agent, e.g., ENS-3029 and a hydrocarbon solvent, e.g., Exxon 5100 N, which encapsules microscopic droplets of an aqueous solution. The components, in the right proportions, are added dropwise to the aqueous solution and mixed with a low to moderate energy input. Addition of a second solution leads to capsule formation, wherein the original solution is contained by liquid

membranes and the second solution is external to the liquid membranes. Stability of the capsules is dependent on the formulation and conditions of generation of the liquid membranes.

By encapsulating a 1 ml of bacterial suspension (coliforms that have been induced and treated with the fluorogenic substrate) into 20  $\mu$ m diameter liquid membrane capsules, all the coliforms should be encapsulated. Fluorescein will be concentrated within the capsules and should fluoresce when exposed to the excitation wavelength. If all the capsules can be counted within a flow fluorocytometer the need for a concentration step is eliminated.

Requirements for liquid membrane encapsulation in the rapid coliform detection procedure include:

- 1) Capsules should be in the 20  $\mu$ m diameter size range,
- 2) Liquid membrane should be impermeable to fluorescein,
- 3) Liquid membrane material must transmit light within the 490 to 550 nm wavelength,
- 4) Capsules should be stable for approximately 1 hour, and
- 5) Materials used in the process must not be pollutants
- 6) Liquid membranes should have a different refractive index than water, if light scattering detection is desired in addition to fluorescence detection.

Liquid membrane capsule formation was investigated. Homogenizer setting, time of homogenization, formulation and charge distribution in the suspending solution were found to be important. Our initial capsules were unfortunately found to fluoresce when observed under the standard operating conditions of the fluorescence microscope.

It was determined that the cause of the interfering fluorescence was the membrane strengthener, a nonionic polyamine material. A new, non fluorescing membrane strengthener, will have to be found before additional work is expended on this approach.

#### Validation for the Rapid Coliform Procedure

No significant progress was made with the validation of the applicability of the rapid coliform detection procedure to field-collected samples. Adverse weather conditions and a long delay in receiving the continuous flow centrifuge was responsible for this lack of progress. Preliminary analyses of cesspool waste, and sewage treatment plant effluent for total and fecal coliform bacteria revealed low numbers (order of  $10^5$  and  $10^4$  coliforms per ml respectively). Because of this difference in total and fecal coliform numbers, we

checked the reliability of the multiple-tube fermentation technique with lactose and lauryl tryphose broth incubated at 35 and 44.5°C. Counts on a cell suspension of laboratory-cultured E. coli were virtually identical based on the total and fecal coliform enumerations. The state of "repair" of the coliforms in the field samples was investigated by comparing counts on selective solid media (McConkey's agar and Eosin methylene blue agar) and MPN lactose broth tubes. Inexplicably, the counts were consistently higher on the selective media than in the broth tubes.

## SUMMARY AND CONCLUSIONS

The procedure outlined in the original feasibility study was extended from total to fecal coliforms by the use of an elevated incubation temperature i.e., 44.5° rather than 35°C. Concentration procedures investigated that held promise of achieving quantitation to the 2 coliform per ml level were the use of a stirred cell concentrator assembled with a Nuclepore membrane (pore size 0-2  $\mu$ m diameter) and a continuous flow centrifuge in conjunction, with a semiautomated fluorescence microscope. Both concentration procedures had the potential of meeting a 100 to 1000 fold concentration goal, while the modified microscope could count 1000 droplets within 20 minutes, lowering the detection level to 5-10 coliforms per ml. However, either concentration step tends to reduce count accuracy and increase the time to run the coliform-counting procedure.

Coliform bacteria incubated with FDG exhibited an intracellular accumulation of fluorescein molecules. Preliminary experiments demonstrated that the resultant fluorochromasia may be sufficient to aid in the detection of individual coliform bacteria on autofluorescence-quenched polycarbonate membrane filters. Coliforms appeared to fluoresce within microdroplets when viewed with the fluorescence microscope. Further work is required before fluorochromasia can be exploited to detect coliform bacteria.

The use of liquid membrane encapsulation of FDG hydrolyzing coliform bacteria, to produce fluorescent capsules that would be detected in a flow cytometer, was briefly investigated. A component of all of the membrane formulations investigated was fluorescent, hence it obscured the excitation wavelengths of fluorescein.

Validation of the rapid coliform detection procedure for counting total and fecal coliforms in field samples was not completed due to premature shut off of funds.

Progress was made in the prefiltration process to remove particulates from the field samples, and the parameters for the optimal induction of coliforms within the field samples were investigated.

Even though the research effort was cut short due to FY 1979 funding difficulties in the Naval Materiel Command, sufficient progress has been made in developing the fluorometric rapid coliforms detection procedure to state that there is a higher probability that the primary objective of detecting and quantifying fecal coliform bacteria at the 2 per ml level in less than one (1) hour can be met.

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